

High resolution melting analysis (HRM) as a new tool for the identification of species belonging to the *Lactobacillus casei* group and comparison with species-specific PCRs and multiplex PCR.

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Abstract

26 The correct identification and characterisation of bacteria is essential for several reasons: the
27 classification of lactic acid bacteria (LAB) has changed significantly over the years, and it is
28 important to distinguish and define them correctly, according to the current nomenclature,
29 avoiding problems in the interpretation of literature, as well as mislabelling when probiotic
30 are used in food products. In this study, species-specific PCR and HRM (high-resolution
31 melting) analysis were developed to identify strains belonging to the *Lactobacillus casei*
32 group and to classify them into *L. casei*, *L. paracasei* and *L. rhamnosus*. HRM analysis
33 confirmed to be a potent, simple, fast and economic tool for microbial identification.
34 In particular, 201 strains, collected from International collections and attributed to the *L. casei*
35 group, were examined using these techniques and the results were compared with
36 consolidated molecular methods, already published. Seven of the tested strains don't belong
37 to the *L. casei* group. Among the remaining 194 strains, 6 showed inconsistent results, leaving
38 identification undetermined. All the applied techniques were congruent for the identification
39 of the vast majority of the tested strains (188). Notably, for 46 of the strains, the identification
40 differed from the previous attribution.

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43 **Keywords:** *Lactobacillus casei* group, High Resolution Melting Analysis, Identification

44 methods, multiplex PCR, species-specific PCR.

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51 **1. Introduction**

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53 Lactic acid bacteria (LAB) are important for the food industry because they promote human
54 health and have therefore been the focus of recent studies (Iqbal et al., 2014). These
55 microorganisms are extremely widespread in nature and are characteristic of many habitats:
56 the gastro-intestinal tracts of various animals such as mice, rats, pigs, chickens and humans;
57 milk and dairy products; fish products; fermented products; and the surfaces of certain plants
58 and fruits. LAB are used in the production and preservation of food products such as cheese,
59 sauerkraut, meat and yogurt (Konings et al., 2000; Settanni and Moschetti, 2010; Shibby and
60 Mishra, 2013; Rubio et al., 2014; Han et al., 2014; Corbo et al., 2014; Beganović et al., 2011,
61 2014; Mani-López et al., 2014). Their important impact on fermented foods and intestinal
62 microflora is due to their antagonistic activity against potential pathogens (de Vrese and
63 Marteau, 2007; Ortolani et al., 2010; Aguilar et al., 2011).

64 The Genus *Lactobacillus* spp. have been extensively studied because of several factors: the
65 importance of these microorganisms in human health; their use in improving the quality or
66 health aspects of many foods; and queries by legislative bodies, industry and consumers about
67 safety, labelling, patents and strain integrity (Shu et al., 1999; Holzapfel and Schillinger,
68 2002; Singh et al., 2009; Doherty et al., 2010; Giraffa et al., 2010; Crittenden, 2012; Harrison
69 et al., 2012; Chen et al., 2014; Didari et al., 2014; El-Abbadi et al., 2014; Fijan, 2014).

70 *Lactobacillus* spp. includes the *L. casei* group, which consists of *Lactobacillus casei*, *L.*
71 *paracasei* and *L. rhamnosus*; these species are used in various commercial and traditional
72 fermented foods. These three species are closely genetically related to each other (Holzapfel
73 and Schillinger, 2002; Ong et al., 2007; Sakai et al., 2010).

74 Recently, the classification of these bacteria has changed considerably because it is difficult to
75 discriminate between *L. casei*, *L. paracasei* and *L. rhamnosus*. However, this distinction is
76 important to understand the relationship between strains, to monitor the genetic stability of the
77 strains and to classify them into recognisable species based on the current taxonomy of these

78 organisms. Furthermore, because of their industrial importance, accurate taxonomic
79 identification of these microorganisms is essential to generate accurate labels for food
80 products and probiotics (Desai et al., 2006).

81 Studies on the 16S rRNA genes of *L. casei*, *L. paracasei* and *L. rhamnosus* revealed that these
82 microorganisms may have minor differences (polymorphisms) even within the same species,
83 which complicates phylogenetic analyses, especially for closely related species (Vásquez et
84 al., 2005).

85 Several techniques have been used to identify and characterise *Lactobacillus* spp. isolates
86 based on their physiological characteristics; these techniques include the study of the
87 fermentative pathways, assays on carbohydrates, lactic acid configuration or peptidoglycan
88 analysis. However, because of the strong similarities, the results of such analyses are often
89 ambiguous (Richiard et al., 2001; Dubernet et al., 2002; Huang et al., 2011); therefore, other
90 studies have focused on genetic characterisation using molecular methods (Klijn et al., 1991;
91 Nuor, 1998; Baele et al., 2002; Comi et al., 2005; Huang and Lee, 2011; Turkova et al., 2012;
92 Salvetti et al., 2012).

93 This study developed and optimised two molecular techniques, high-resolution melting
94 (HRM) analysis and species-specific PCRs, to identify species belonging to the *L. casei*
95 group. A large number of strains (201), taxonomically indicated as *L. casei*, *L. paracasei* and
96 *L. rhamnosus*, were obtained from International Collections and subjected to a series of novel
97 trials for accurate identification using two consolidated molecular methods described
98 previously. These results were compared to the results obtained using the species-specific
99 PCR and HRM analyses developed in this study.

100

101 **2. Materials and methods**

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103 *2.1. Strains and culture conditions*

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105 Two hundred one (201) strains belonging to the species *Lactobacillus casei*, *L. paracasei* and
106 *L. rhamnosus* isolated from different sources (Table 1) were used in this study. The strains
107 were previously isolated and identified by the respective Universities or Research Institutes
108 using biochemical and morphological tests or different molecular techniques.

109 All strains were maintained as frozen stocks in reconstituted 11 % (w/v) skimmed milk
110 containing 0.1 % (w/v) ascorbic acid (RSM) in the Culture Collection of the Department of
111 Food Science, University of Udine. The isolates were routinely propagated (1 % w/v) in MRS
112 broth (pH 6.8) (Oxoid, Milan, Italy) for 16 h at 37 °C.

113 *L. casei* (DSM 20178), *L. paracasei* (DSM5622) and *L. rhamnosus* (DSM20021) were used
114 as reference strains for optimisation of all the molecular methods used for identification. The
115 following strains were used as negative controls: *Lactobacillus fermentum* (DSM 20049), *L.*
116 *pontis* (DSM 8475), *L. sanfranciscensis* (DSM 20451), *L. brevis* (DSM 20054), *L. reuteri*
117 (DSM 20053), *L. plantarum* (DSM 20174), *L. sakei* (DSM 6333), *Lactococcus lactis* (DSM
118 20481), *Leuconostoc citreum* (DSM 5577), *Leuc. gasicomitatum* (DSM 15947), *Leuc.*
119 *mesenteroides subsp. mesenteroides* (DSM 20343) and *Pediococcus pentosaceus* (DSM
120 20336).

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122 2.2. DNA extraction from pure cultures

123

124 Two millilitres of a 48-h culture in De Man-Rogosa-Sharp (MRS) broth were centrifuged at
125 13,000g for 10 minutes at 4 °C to pellet the cells, which were then subjected to DNA
126 extraction using the MasterPure™ Complete DNA & RNA Purification Kit (Epicentre
127 Biotechnologies, USA). The DNA concentration and purity were measured using an
128 absorbance ratio of 260/280 nm and verified by agarose gel electrophoresis.

129

130 2.3. *L. casei* group-specific PCR

131

132 The *L. casei* group-specific PCR primer pair, LCgprpoA-F2 (5'-
133 CACTCAARATGAAYACYGATGA-3') and -R2 (5'-CGTGGTGAGATTGAGCCAT-3')
134 was used as described by Huang et al. (2011). The reactions were performed in a final volume
135 of 25 µl containing 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM dNTPs,
136 0.2 mM of each primer and 1.25 U of *Taq*-polymerase (Applied Biosystems, Milan, Italy).
137 The thermal cycling protocol was as follows: initial strand denaturation at 94 °C for 5 min
138 followed by 25 cycles of 94 °C for 1 min, 61 °C for 1 min and 72 °C for 1.5 min, and a final
139 extension step at 72 °C for 7 min in a Thermal Cycler (DNA Engine Dyad Peltier Thermal
140 Cycler, BioRad, Milan, Italy). The PCR products were analysed by 2 % agarose gel
141 electrophoresis with ethidium bromide staining, and the expected amplicon size was 364 bp.

142

143 2.4. Species-specific PCRs

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145 Three different primer pairs were used to identify strains by species-specific PCRs, as
146 described by Ward and Timmins (1999) (Table 2). The reactions were performed in a final
147 volume of 25 µl containing 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM
148 dNTPs, 0.2 mM of each primer and 1.25 U of *Taq*-polymerase (Applied Biosystems, Milan,
149 Italy). The amplification was performed for 30 cycles at 95 °C for 1 min, 53 °C for 1 min and
150 72 °C for 1 min in a Thermal Cycler (DNA Engine Dyad Peltier Thermal Cycler, BioRad,
151 Milan, Italy). An initial denaturation step (95 °C for 5 min) and a final extension step (72°C
152 for 5 min) were used. The PCR products were verified by electrophoresis in a 2 % agarose gel
153 using 0.5X TBE as the running buffer. Ethidium bromide (0.5 µg/ml) was added to the gel
154 before solidification. After electrophoresis, the gels were examined using the BioImaging
155 System GeneGenius (*SynGene*, Cambridge, United Kingdom).

156 In this study, a second set of species-specific PCRs was developed, using a different part of
157 the genome as a target sequence for primer annealing compared to the region used by Ward
158 and Timmins (1999). The *dnaJ* and *dnaK* genes were targeted. All of the sequences of these
159 genes available in GenBank for species of the *L. casei* group were aligned using the MultAlin
160 software (Corpet, 1988), and the primer pairs designed were dnaKRHf/dnaKRHr,
161 dnaKCPf/dnaKCPPr, and dnaJCPf/dnaJCPPr (Table 2), which were specific to the *L. casei*
162 group for *L. rhamnosus*, *L. paracasei*/*L. casei* and *L. paracasei*, respectively. Before
163 optimisation of the amplification protocol, primer specificity was tested *in silico* using the
164 FastPCR 6.1 software (Kalendar et al., 2009) and *in vivo* using *Lactobacillus fermentum* (DSM
165 20049), *L. pontis* (DSM 8475), *L. sanfranciscensis* (DSM 20451), *L. brevis* (DSM 20054), *L.*
166 *reuteri* (DSM 20053), *L. plantarum* (DSM 20174), *L. sakei* (DSM 6333), *Lactococcus lactis*
167 (DSM 20481), *Leuconostoc citreum* (DSM 5577), *Leuc. gasicomitatum* (DSM 15947), *Leuc.*
168 *mesenteroides subsp. mesenteroides* (DSM 20343) and *Pediococcus pentosaceus* (DSM 20336) as
169 negative controls.

170 The reactions were performed in a final volume of 25 µl containing 10 mM Tris HCl (pH 8.3),
171 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.2 mM of each primer and 1.25 U of *Taq*-
172 polymerase (Applied Biosystems, Milan, Italy). PCR was performed using the thermal
173 cycling protocol described above, with the annealing temperatures shown in Table 2.

174

175 2.5. *tuf* multiplex PCR

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177 Amplification reactions were performed with a 50 µl (total volume) solution containing 10
178 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, 200 µM each dNTP, 10 pmol each of primers
179 PAR (5'-GACGGTTAAGATTGGTGAC-3'), CAS (5'-ACTGAAGGCGACAAGGA-3'),
180 and RHA (5'-GCGTCAGGTTGGTGTG-3'), 50 pmol of primer CPR (5'-
181 CAANTGGATNGAACCTGGCTTT-3') (Ventura et al., 2003), 25 ng of template DNA, and

182 2.5 U of *Taq*-DNA polymerase (Applied Biosystems, Milan, Italy). Amplification reactions
183 were performed using a thermocycler (Perkin-Elmer Cetus 9700) with the following
184 temperature profiles: 1 cycle at 95 °C for 5 min; 30 cycles at 95 °C for 30 s, 54 °C for 1 min,
185 and 72 °C for 1.5 min; and 1 cycle at 72 °C for 7 min, in a Thermal Cycler (DNA Engine
186 Dyad Peltier Thermal Cycler, BioRad, Milan, Italy). PCR amplicons were analysed by 2%
187 (w/v) agarose gel electrophoresis in TBE 0.5X buffer at a constant voltage of 7 V/cm,
188 visualised with ethidium bromide (0.5 µg/ml), and photographed under UV light at 260 nm,
189 using the BioImaging System GeneGenius (*SynGene*, Cambridge, United Kingdom).

190

191 2.6. Development and optimisation of High-Resolution Melting (HRM) analysis

192

193 The variable regions V1 to V3 flanked by highly conserved sequences within the 16S rRNA
194 were selected for HRM analysis. Three consolidated primer pairs that have been used to
195 discriminate different species by DGGE analysis were used to discriminate *L. casei*, *L.*
196 *paracasei* and *L. rhamnosus* by HRM analysis: P1V1 and P2V1 (Klijin et al., 1991), BA-338f
197 and UN-518r (Muyzer et al., 1993), Y1 and Y2 (Young et al., 1991). The analyses were
198 performed in a 25-µl reaction volume containing 2X HRM PCR Master mix (Qiagen, Milan,
199 Italy), 0.7 µM each primer and 100 ng of DNA. The PCR amplifications were performed in a
200 Rotor-Gene Q (Qiagen, Milan, Italy) with the following conditions: 95 °C for 1 min followed
201 by 45 cycles at 95 °C for 10 s, 55 °C for 30 s 72 °C for 10 s. After amplification, HRM
202 analysis was performed from 65 to 90 °C with increments of 0.1 °C/2 sec. The Rotor-Gene Q
203 series software version 2.2.2 (Qiagen, Milan, Italy) was used to analyse the HRM data. The
204 melting profiles were subjected to fluorescence normalisation to minimise inter- and intra-run
205 variability. Difference plots were generated by normalising the melting profiles of strains to a
206 negative control strain whose melting profile was converted to a horizontal line. Three
207 difference graphs were obtained for the *L. casei*, *L. paracasei* and *L. rhamnosus* strains using

208 the fluorescence of each reference strain (per each graph) set as the baseline (confidence level
209 of 90 %) (Andersson et al., 2009; Gurtler et al., 2012). The ScreenClust program (Qiagen,
210 Milan, Italy) was used for Principal Component Analysis (PCA).

211 All analyses were performed in triplicate; positive/negative controls and non-template
212 controls (NTC) were included in each run. For the validation assay, 10 strains were used for
213 each species tested.

214

215 **3. Results and discussion**

216

217 *3.1 Preliminary identification by L. casei group-specific PCR*

218

219 A total of 201 strains belonging to the *L. casei* group were collected from national and
220 international collections (Table 1). The strains were isolated from sources including raw and
221 heat-treated milk, yogurt, milking machines, green/creamy and seasoned cheeses, fermented
222 sausages, sourdoughs, wine, must and cellar equipment, beer, malt, coffee and humans; the
223 source of some strains was unknown. These strains were isolated over several years.
224 Therefore, in some cases, there was no information on the origin or method of identification
225 used. In other cases, biochemical tests or molecular analyses were performed for strain
226 identification. To uniformly identify strains, a preliminary *L. casei* group-specific PCR was
227 performed. The expected amplicon was obtained from 194 strains (Figure 1), confirming that
228 these strains belonged to the *L. casei* group. The amplicon was not obtained from 7 strains,
229 and they were excluded from subsequent analyses. None of the negative control strains
230 yielded the amplicon, confirming the specificity of the primers.

231

232 *3.2. Species identification by species-specific PCRs and tuf multiplex PCR*

233

234 The identification methods were tested on the three reference strains and were able to
235 discriminate *L. casei*, *L. paracasei* and *L. rhamnosus* species (Figure 2). Species-specific
236 PCRs yielded an amplicon of the expected size (290 bp) only for the target species, and no
237 amplification product was obtained for the other two *L. casei* group species (Figure 2, panel
238 A). Similarly, the *tuf* multiplex PCR profiles yielded different numbers of bands for *L. casei*,
239 *L. paracasei* and *L. rhamnosus*, which enabled the discrimination of these species. The
240 amplification profile of *L. casei* comprised five bands of approximately 350, 450, 500, 900
241 and 1100 bp, which was not completely consistent with the profile obtained by Ventura *et al.*
242 (2003). The *L. paracasei* amplification profile comprised a strong band of approximately 200
243 bp and a thinner band of 500 bp, which was not always visible (Figure 2, panel B, lines L5
244 and L10); Ventura *et al.* (2003) obtained strong amplification products corresponding to these
245 sizes. The amplification profile of *L. rhamnosus* comprised a single amplicon of
246 approximately 500 bp, consistent with Ventura *et al.* (2003). Although both these techniques
247 discriminated species within the *L. casei* group, amplification products were also obtained for
248 specific negative control LAB strains (data not shown); these strains yielded a 290-bp
249 amplicon in the species-specific PCR analysis and profiles comparable to the *L. casei* group
250 species in the *tuf* multiplex PCR analysis. Therefore, a preliminary screening step comprising
251 the *L. casei* group-specific PCR is required for the identification of LAB isolates using these
252 techniques.

253 Inconsistent results were obtained only for 6 out of the 194-tested *L. casei* group strains using
254 the two techniques (Table 3) and, for some of them (2), the obtained results were not
255 unexpected. In fact, LMG6904 (synonyms ATCC393, DSM20011, CCUG21451) is a well-
256 known strain whose taxonomic classification has been repeatedly modified and is under
257 debate; the Judicial Commission of the International Committee for Systematics of
258 Prokaryotes ruled the following: i) The designation of ATCC334, a strain of *L. paracasei*, as
259 the neotype of *L. casei* contravenes rules 51b (1) and (2); ii) Typification of *L. casei* (Orla-

260 Jensen 1916) Hansen and Lessel 1971 is based on ATCC393; iii) The proposal to revive the
261 name *L. zeae* contravenes rules 51b (1) and (2); iv) The name *L. paracasei* has not been
262 rejected by the Judicial Commission and is legitimate, validly published and may be used as a
263 correct name. This ruling confirms the deliberations (Wayne, 1994) that followed a previous
264 Request for Opinion by Dellaglio et al. (1991) (Dellaglio et al., 1991; Waine, 1994; Dicks et
265 al., 1996; Mori et al., 1997; Chen et al., 2000; Biavati, 2001; Klein, 2001; Dellaglio et al.,
266 2002; Judicial Commission Of The International Committee On Systematics Of Prokaryotes,
267 2008). Identification of the strain DSM4905 (synonym ATCC1158) is also ambiguous based
268 on the species classification provided by the DSM and ATCC collections. In the DSM
269 collection, this strain is considered as the reference strain for the *L. paracasei* species,
270 whereas the ATCC considers this strain as the reference strain for the *L. casei* species. The
271 taxonomic classification of these two strains, as well as the remaining four strains out of the 6,
272 (DBPZ0420, DBPZ0571, DBPZ0734 and N2014) requires further studies.

273 For the other 188 strains out of the 196, the two identification methods yielded consistent
274 results, but for 46 out of the 188 strains, the results were in disagreement with the original
275 identification.

276 To confirm these results, two different methods were developed in this study: alternate
277 species-specific PCRs and HRM analysis.

278 The species-specific primer pairs designed for the *dnaK* and *dnaJ* genes were specific within
279 the *L. casei* group; amplicons were obtained exclusively from *L. rhamnosus*, *L. paracasei*/*L.*
280 *casei* and *L. casei* using the primer pairs dnaKRHf/dnaKRHr (Figure 2, panel C, a),
281 dnaKCPf/dnaKCPr (Figure 2, panel C, b) and dnaJPAf/dnaJPAr (Figure 2, panel C, c),
282 respectively. All the 194 strains, belonging to the *L. casei* group, were tested. The results were
283 consistent with the species-specific PCRs and *tuf* multiplex PCRs for the 188 strains. The data
284 for the 6 unidentified strains LMG6904, DSM4905, DBPZ0420, DBPZ0571, DBPZ0734, and
285 N2014 are shown in Table 3.

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287 3.2. High-resolution melting (HRM) analysis

288

289 HRM analysis was used to resolve inconsistencies between the species-specific PCR and *tuf*
290 multiplex PCR analyses compared to the original identification.

291 HRM analysis is a novel technique that enables the identification of point mutations in a DNA
292 sequence. It has been previously used to characterize nonstarter lactic acid bacteria (Porcellato
293 et al., 2012a, 2012b), and the results seemed to be promising in discriminating among the *L.*
294 *casei* group species. This technique involves the amplification of a specific DNA sequence
295 using a primer pair that allows annealing and DNA amplification in all the three species
296 considered. The amplicons were produced using the qPCR technique and SYBR Green as an
297 intercalating fluorescent dye and then subjected to a thermal gradient with temperature
298 increments of 0.1 °C/sec using sensitive instrumentation that enables absolute precision of the
299 temperatures used. By continuously monitoring the fluorescence emitted by SYBR Green, it
300 is possible to assess the exact melting temperature of the amplicon, with a precision of 0.1 °C.
301 Base differences and/or insertions or deletions of one or more bases is revealed, and this
302 enables discrimination between amplicons and, consequently, between species.

303 Before using HRM analysis, a preliminary optimisation step was performed to determine the
304 most effective primer pair among three candidate pairs. For optimisation, six strains whose
305 original identification was confirmed by both species-specific PCRs and *tuf* multiplex PCRs
306 were used: *Lactobacillus casei* DSM20178 and LACcas7; *Lactobacillus rhamnosus*
307 DSM20021 and 2220; *Lactobacillus paracasei* DSM20258 and DSM5622. HRM analysis on
308 these strains revealed that only the primers P1V1-P2V1 were effective in discriminating
309 among the three species (Figure 3). The primer pairs BA-338f/UN518r and Y1/Y2 yielded
310 amplicons with highly similar melting curves comprising the following melting peaks:
311 DSM20178 *L. casei*, 85.95 °C; DSM5622 *L. paracasei*, 85.55 °C; DSM20258 *L. paracasei*,

312 85.38 °C; 2220 *L. rhamnosus*, 85.47 °C; DSM20021 *L. rhamnosus*, 85.40 °C; LACcas7 *L.*
313 *casei*, 84.30 °C (using BA-338f/UN518r) and DSM20178 *L. casei*, 84.90 °C; LACcas7 *L.*
314 *casei*, 86.40 °C; DSM5622 *L. paracasei*, 84.85 °C; 2220 *L. rhamnosus*, 84.30 °C; DSM20021
315 *L. rhamnosus*, 84.30 °C; DSM20258 *L. paracasei*, 84.67 °C (using Y1/Y2). Considering these
316 data and the melting curves (Figure 3, panel A, a; panel B, a), the normalised melting curves
317 (Figure 3, panel A, b; panel B, b) and the principal component analysis (PCA) graphs (Figure
318 3, panel A, c; panel B, c), these primer pairs could not be used to discriminate among the
319 three species. However, the melting profiles and the normalised fluorescence curves as well as
320 the PCA of the amplicons obtained using P1V1/P2V1 allowed to group the strains into 3
321 species-specific clusters (Figure 3, panel C, a, b, and c).

322 To examine the reproducibility of these data, HRM analysis was performed on five replicates
323 for each strain, and the curves overlapped completely. The average melting temperature of the
324 standard strains tested was 83.69 ± 0.03 °C for *L. casei* (DSM20178 and LACcas7, 5
325 replicates per strain); 81.66 ± 0.06 °C for *L. rhamnosus* (DSM20021 and 2220, 5 replicates
326 per strain), and 84.16 ± 0.04 °C for *L. paracasei* (DSM20258 and DSM5622, 5 replicates per
327 strain). Therefore, HRM analysis yielded reproducible results. To highlight the differences
328 among the three species, 3 difference graphs were generated using *L. casei* (DSM20171), *L.*
329 *paracasei* (DSM20258) and *L. rhamnosus* (DSM20021) (confidence level of 90 %) as
330 baselines (Figure 4, panel A, B, and C).

331 The blue, green and pink curves indicate ten replicates of the two *Lactobacillus rhamnosus*, *L.*
332 *casei* and *L. paracasei* strains, respectively. When one species was used as the baseline, the
333 fluorescence values for that species were almost a flat line, whereas the other two species had
334 different performance curves. These graphs indicate the difference in the amplitudes of the
335 curves and that this technique clearly discriminated the three species. Furthermore, the
336 replicates yielded overlapping normalised curves, confirming the reproducibility of this
337 technique. The different graph amplitudes are derived from melting curves that are always

338 normalised to the same number of arbitrary fluorescence units by the Corbett 6000 software;
339 therefore, these amplitudes can be compared across different runs using *L. casei* (DSM20178),
340 *L. paracasei* (DSM20258) and *L. rhamnosus* (DSM20021) as standard controls in each run.
341 After optimisation of HRM analysis, all the 194 strains, confirmed to belong to the *L. casei*
342 group, were analysed using this method. Because of the large number of strains, more runs
343 were required, and standard controls were included to reveal any changes and to compare all
344 the tested strains at the end of the analysis. Therefore, after PCA, it was possible to identify
345 the strains according to the cluster in which they were grouped (Figure 5). The example
346 shown in Figure 5 demonstrates that the three species were grouped in three well-defined and
347 distant clusters (Figure 5, panel A). The normalised fluorescence curves overlapped
348 completely (Figure 5, panel B); the difference graphs showing the normalised fluorescence
349 curves vs. the control strains, also overlapped completely (Figure 5, panels C). On the basis of
350 the data obtained during the optimization, HRM confirmed to be a potent tool for microbial
351 identification, also considering their advantages: it is a simple, rapid, and inexpensive method,
352 even if depends strongly on good PCR instruments and dyes. Moreover, there is no need to
353 process the sample after the PCR reaction, and this allows to increase the sensitivity of the
354 method in respect to a traditional PCR, followed by agarose gel electrophoresis; it allows the
355 detection and, using appropriate standard curves, also the quantification of several genotypes
356 in qPCR reactions with a single primer pair, in a unique reaction, as performed by Lin and
357 Gänzle (2014). The results of the HRM analysis were consistent with the other methods used
358 in this study, confirming the identity of 188 strains; inconsistent results were obtained only for
359 the 6 strains shown in Table 3. Further studies such as whole-genome sequencing are required
360 to elucidate the taxonomic classification of these strains. For 46 of the remaining 188 strains,
361 the strain identity obtained using this method was inconsistent with the original identification
362 (Table 4). Notably, the strain DIALYac was isolated from a commercial probiotic yogurt and
363 identified as *L. casei* (Shirota); however, in this study, all methods classified this strain as *L.*

364 *paracasei*, accordingly to with Sutula et al. (2012). Therefore, there is significant ambiguity in
365 the use of the correct taxonomic name in industrial and scientific settings. In fact, also in
366 recent studies the old classification name has been used (Douillard et al., 2013).

367

368 **4. Conclusions**

369

370 Accurate strain classification is critical for strains that are important for industrial purposes,
371 including strains belonging to the *L. casei* group, which have probiotic properties. There is
372 significant ambiguity in strain names within the *L. casei* group because some authors use the
373 new classification system (Dellaglio et al., 2002; Dobson et al., 2004), whereas others do not
374 (Mori et al., 1997; Ward and Timms, 1999; Vásquez et al., 2005; Desai et al., 2006).

375 Furthermore, commercial strains are often described as “*L. casei*”, and this description is used
376 for strains of any of these species. Furthermore, these species share close genetic relationships,
377 and accurate identification is difficult (Nuor, 1998; Beale et al., 2002; Klijn et al., 1991). The
378 use of multiple coupled techniques can elucidate the taxonomic position of some strains;
379 therefore, we proposed two new molecular tools to identify species belonging to the *L. casei*
380 group: species-specific PCRs and HRM analysis. Both methods yielded accurate results, and
381 considering the large number of strains tested (194), these methods were effective in
382 discriminating among the three species within the *L. casei* group. For some strains, the results
383 obtained using these methods were inconsistent with the original identification and the results
384 obtained using other molecular methods. This discrepancy is not unexpected because in most
385 cases, the original identification was performed using phenotypical and biochemical tests.
386 These tests are often based on colour changes, which can be misinterpreted because colour
387 changes are rarely precise and sharp. Misinterpretation of these results often leads to an
388 incorrect identification. Furthermore, many strains were identified at a time when only one

389 species, *L. casei*, and the subsp. *paracasei* were classified. Therefore, the classification of
390 these strains was not consistent with the current strain taxonomy.

391

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395

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642 **Figure legends.**

643

644 **Figure 1. Specific PCR for *Lactobacillus casei* group** (amplicon size 364 bp): line 1: ladder,
645 100 bp low ladder (Sigma-Aldrich, Milan, Italy); line 2: DSM20178, *L. casei*; line 3:
646 DSM20021, *L. rhamnosus*; line 4: DSM5622, *L. paracasei*; line 5: DSM 20451, *L.*
647 *sanfranciscensis*; line 6: DSM 20054, *L. brevis*; line 7: DSM 20053, *L. reuteri*; line 8: DSM
648 20174, *L. plantarum*; line 9: DSM 6333, *L. sakei*; line 10: DSM 20481, *Lactococcus lactis*;
649 line 11: DSM 5577, *Leuconostoc citreum*; line 12: DSM 15947, *Leuc. gasicomitatum*; line 13:
650 DSM 20343, *Leuc. mesenteroides subsp. mesenteroides*; line 14: DSM 20336, *Pediococcus*
651 *pentosaceus*; line 15: negative control.

652

653 **Figure 2. Species identification by species-specific PCRs and *tuf* multiplex PCR. Panel A,**
654 Species-specific PCRs by Ward and Timmins (1999). **a)** Amplification specific for *L. casei*; **b)**
655 Amplification specific for *L. paracasei*; **c)** Amplification specific for *L. rhamnsosus*. Line 1, Ladder,
656 100 bp low ladder (Sigma-Aldrich, Milan, Italy); line 2, DSM20178, *L. casei*; line 3, DSM5622, *L.*
657 *paracasei*; line 4, DSM20021, *L. rhamnosus*; NC, negative control. **Panel B**, *tuf* multiplex PCR by
658 Ventura et al. (2003). Lanes L1, L14: Ladder 100 bp (New England Biolabs); Lanes L2, L13: Ladder
659 50 bp (New England Biolabs); Lane L3: DSM20021, *Lactobacillus rhamnosus*; Lane L4: negative
660 control; Lane L5: DSM5622, *Lactobacillus paracasei*; Lane L6: FSG01, *Lactobacillus rhamnosus*;
661 Lane L7: DSM20178, *Lactobacillus casei*; Lane L8: N87, *Lactobacillus casei*; Lane L9: D44,
662 *Lactobacillus rhamnosus*; Lane L10: Cst7, *Lactobacillus paracasei*; Lane L11: N202, *Lactobacillus*
663 *rhamnosus*; Lane L12: N1110, *Lactobacillus rhamnosus*. **Panel C**, Species-specific PCRs, this study.
664 **a)** Amplifican specific for *L. rhamnosus*. Line 1, ladder, 100 bp low ladder (Sigma-Aldrich, Milan,
665 Italy); line 2, negative control; line 3, DSM20021, *L. rhamnosus*; line 4, N202, *Lactobacillus*
666 *rhamnosus*; line 5, DSM20178, *L. casei*; line 6, N87, *Lactobacillus casei*; line 7, DSM5622, *L.*
667 *paracasei*; line 8, Cst7, *Lactobacillus paracasei*. **b)** Amplification specific for *L. paracasei/L. casei*.
668 Line 1, ladder, 100 bp low ladder (Sigma-Aldrich, Milan, Italy); line 2, negative control; line 3,

669 DSM20021, *L. rhamnosus*; line 4, N202, *Lactobacillus rhamnosus*; lines 5-6, DSM20178, *L. casei*;
670 line 7, N87, *Lactobacillus casei*; line 8, DSM5622, *Lactobacillus paracasei*. **c)** Amplification specific
671 for *L. paracasei*. Line 1, ladder, 100 bp low ladder (Sigma-Aldrich, Milan, Italy); line 2, negative
672 control; line 3, DSM20021, *L. rhamnosus*; line 4, N202, *Lactobacillus rhamnosus*; line 5, DSM5622,
673 *Lactobacillus paracasei*; line 6, Cst7, *Lactobacillus paracasei*; line 7, LMG13087, *L. paracasei*; lines
674 8, DSM20178, *L. casei*.

675

676 **Figure 3. HRM results obtained using the three different couples of primers. Panel A,**
677 **primers BA-338f / UN518r; Panel B, primers Y1 / Y2; Panel C, primers P1V1 / P2V1. a)**
678 **Melting curves profiles; b) Normalized melting curves; c) Principal component analysis**
679 **(PCA).**

680

681 **Figure 4. Difference graphs obtained for the ten replicates of the three standard species.**
682 **Panel A)** *L. casei* was used as the baseline; **panel B)** *L. paracasei* was used as the baseline;
683 **panel C)** *L. rhamnosus* was then used as the baseline.

684

685 **Figure 5. HRM analysis of 46 out of the 196 strains.** Panel A, Principal component
686 analysis; panel B, Normalised fluorescence curves; panels C, difference graphs. **Cluster 1,** *L.*
687 *casei*; **cluster 2,** *L. paracasei*; **cluster 3,** *L. rhamnosus*.

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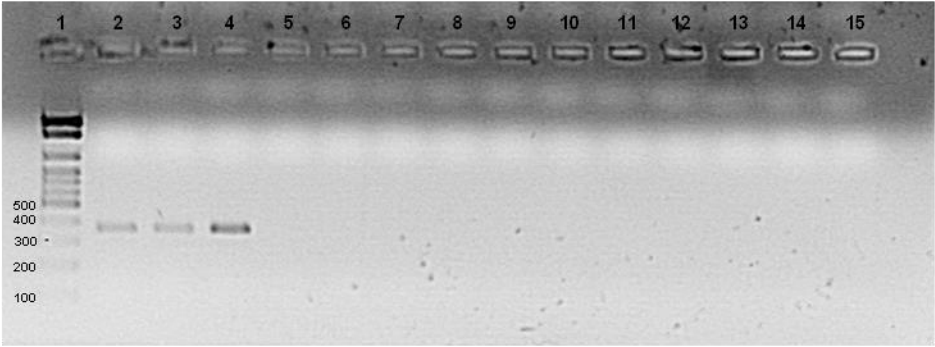
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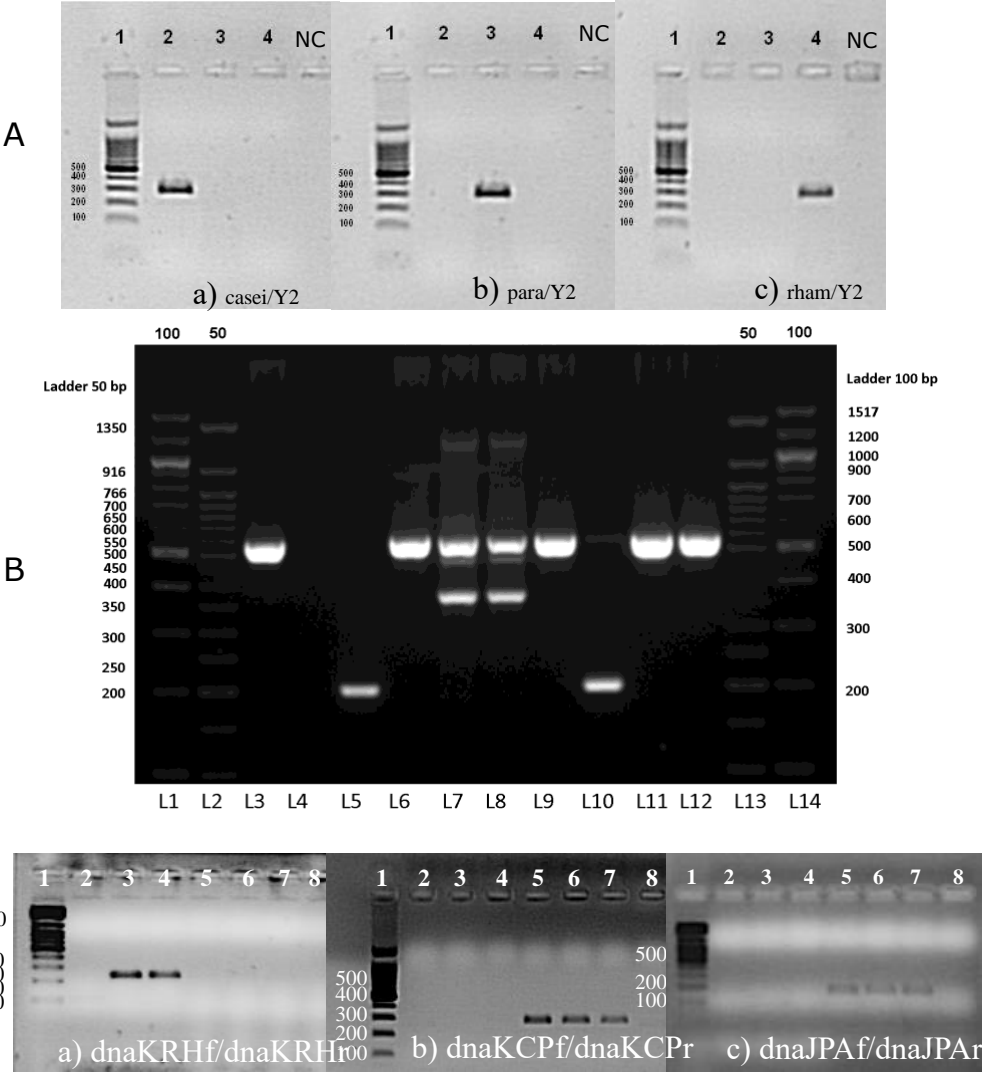
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695 **Figure 1.**
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700 **Figure 2.**



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Figure 3.

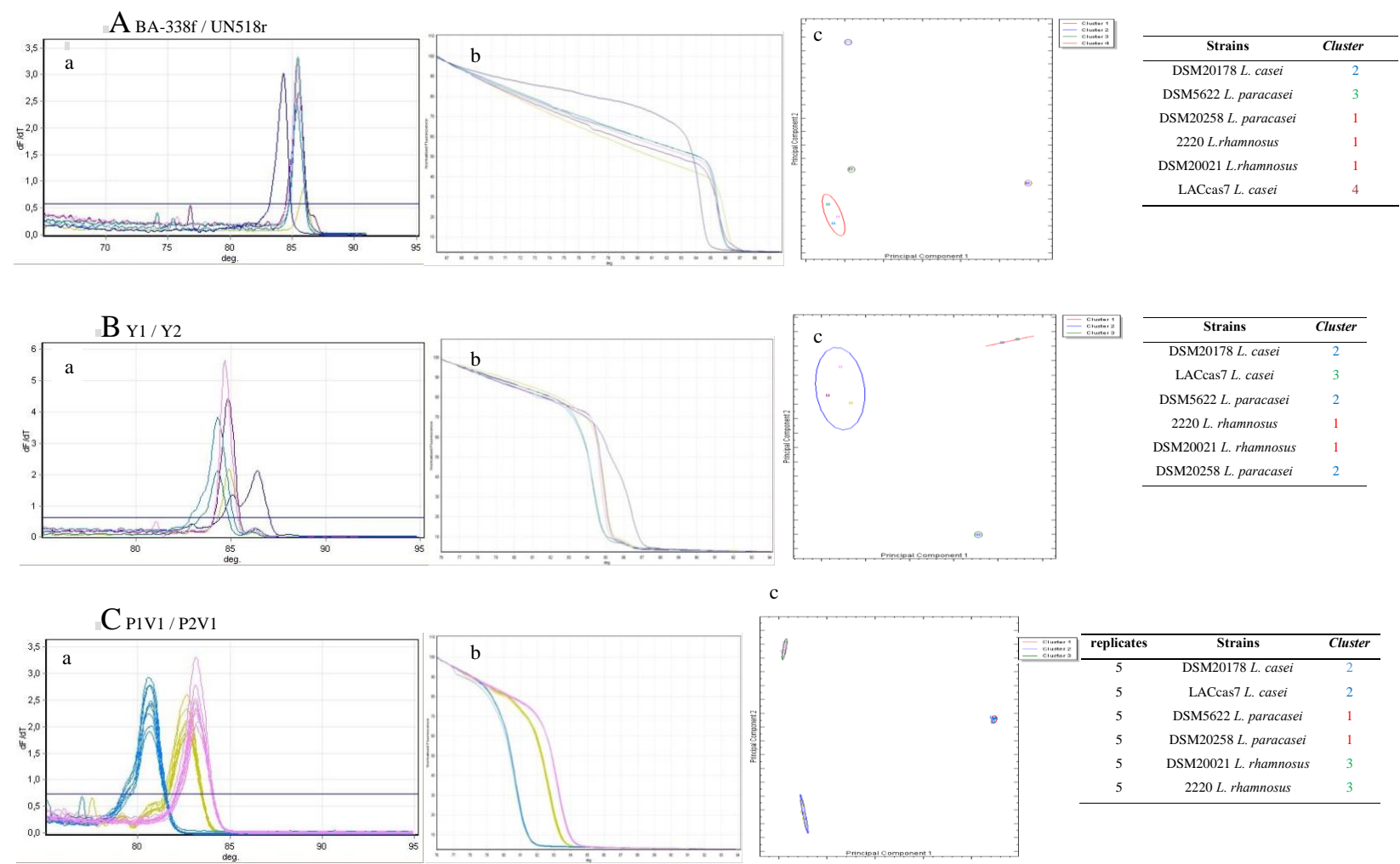


Figure 4.

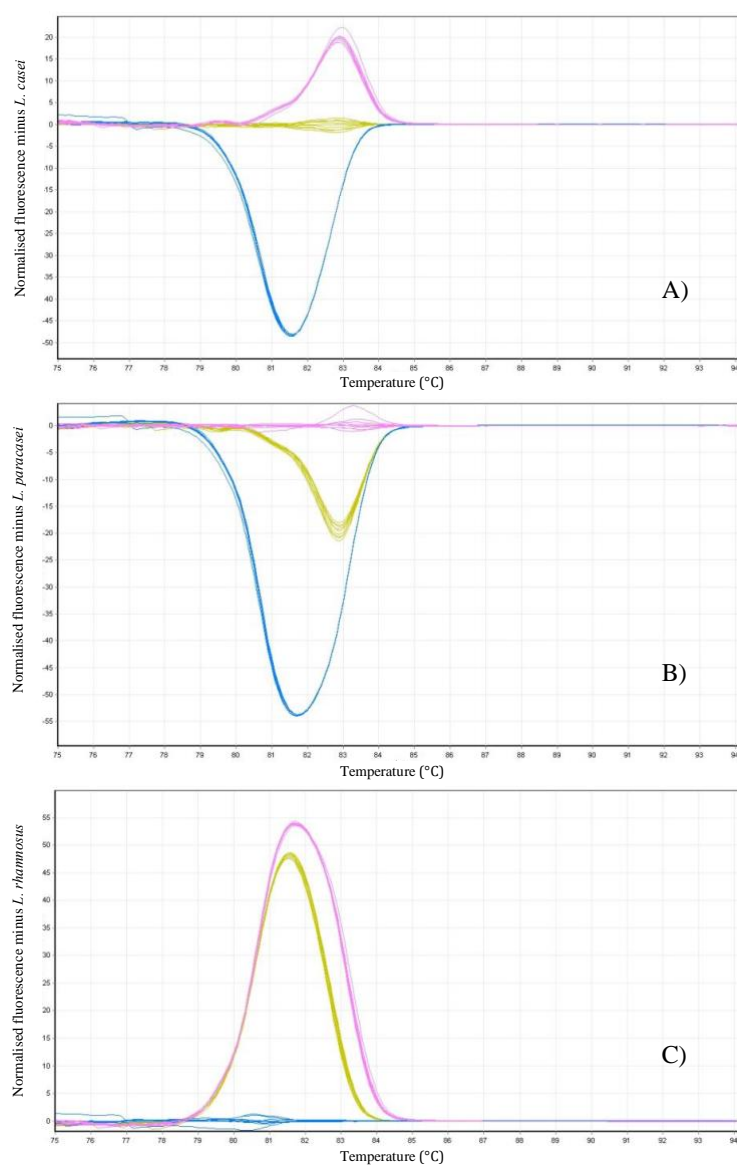


Figure 5.

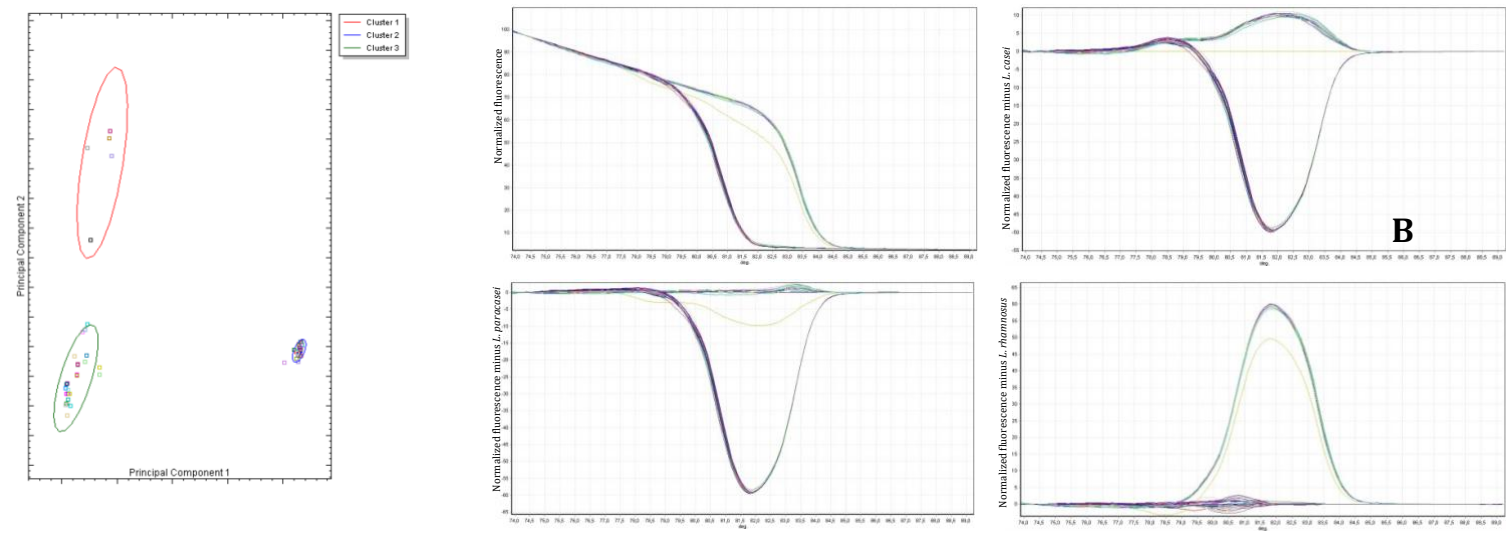


Table 1. Origin and given identification of the 199 strains collected for the study.

Origin	Given identification
Raw and heat treated milk, yoghurt, milking machines	<i>L. paracasei</i> : LMG9192 ₁ , DSM5622 ₂ <i>L. Casei/paracasei</i> : P1E5 ₃ , P1E6 ₃ , P2P3 ₃ <i>L. paracasei subsp. tolerans</i> : LMG9191 ₁ , P1E4 ₃ , DSM20258 ₂ <i>L. rhamnosus</i> : HA111 ₄ , PRA152 ₅ , CI230 ₅
Green, creamy and seasoned cheeses (Italian cheeses: Scamorza, Parmigiano Reggiano, Grana Padano, Sprezza, Asiago, Montasio, Canestrato di Moliterno, Morlacco, Bellunese, Pecorino, Caciocavallo, Provolone, Emmenthal, Raclette de Savoie; Chinese and Tunisian cheeses)	<i>L. casei</i> : LMG6904 ₁ , TMW1.1444 ₆ , TMW1.1259 ₆ , LACcas13 ₇ , LACcas7 ₇ <i>L. paracasei</i> : LMG25880 ₁ , LMG25883 ₁ , LMG12164 ₁ , DBPZ0421 ₈ , DBPZ0422 ₈ , DBPZ0424 ₈ , DBPZ0434 ₈ , DBPZ0435 ₈ , DBPZ0450 ₈ , DBP0451 ₈ , DBPZ0472 ₈ , DBPZ0475 ₈ , DBPZ0476 ₈ , DBPZ0477 ₈ , DBPZ0478 ₈ , DBPZ0635 ₈ , DBPZ0733 ₈ , M266 ₈ , M268 ₈ , M299 ₈ , M308 ₈ , M348 ₈ , M354 ₈ , M359 ₈ , S1 ₈ , S3 ₈ , V3 ₈ , W11 ₈ , DSG03 ₈ , DSG05 ₈ , DSG07 ₈ , ESG10 ₈ , HSG09 ₈ , PSG06 ₈ , PSG09 ₈ , PSG10 ₈ , P71 ₉ , TH1229 ₉ , SP57 ₉ , L24 ₉ , TH406 ₉ , FSL436 ₁₀ , FSL451 ₁₀ , DBPZ0436 ₈ , DBPZ0428 ₈ , M335 ₈ , M290 ₈ , M303 ₈ , H121 ₃ <i>L. casei/paracasei</i> : Cst7 ₁₁ , 3LC1 ₁₁ , DBPZ0718 ₈ , M307 ₈ <i>L. rhamnosus</i> : M15 ₉ , O14 ₈ , PRA204 ₅ , PRA232 ₅ , PRA331 ₅ , DBPZ0430 ₈ , DBPZ0445 ₈ , DBPZ0446 ₈ , DBPZ0448 ₈ , DBPZ0449 ₈ , FSG01 ₈ , CI4362 ₁₂ , CF1350 ₁₂ , CF377 ₁₂ , D441 ₃ , H251 ₃ , 5A9T ₉ , 5D9T ₉ , L9 ₉ , L47 ₉ , CI4368 ₁₂ , DBPZ0420 ₈ , DBPZ0734 ₈ , CF1431 ₂ , R611 ₃ , F171 ₃ , N241 ₃
Fermented sausages	<i>L. casei/paracasei</i> : CTC1675 ₁₄ <i>L. casei/rhamnosus</i> : CTC1676 ₁₄ , 2220 ₁₅
Sourdoughs	<i>L. paracasei</i> : DBPZ0561 ₈ , DBPZ0571 ₈ , DBPZ0572 ₈ , Q2 ₈ , Q4 ₈ , I14 ₄ , I216 <i>L. casei/paracasei</i> : DBPZ0563 ₈ , DBPZ0564 ₈ , DBPZ0579 ₈ , I316
Wine, must and cellar equipment's	<i>L. paracasei</i> : LMG11961 ₁ , LMG11963 ₁ , LMG13717 ₁ , LMG13731 ₁ , B0611 ₇ , B0821 ₇ , B0831 ₇ , B0851 ₇ , B0871 ₇ , B1611 ₇ , B1691 ₇ , B1711 ₇ , B1741 ₇ , B1951 ₇ , B1961 ₇ , B3501 ₇ , B1661 ₇ , B0841 ₇ , B0861 ₇ , B1631 ₇ , B1641 ₇ , B1671 ₇ , B1681 ₇ , B1701 ₇ , B1721 ₇ , B1731 ₇ , B1751 ₇ , B1791 ₇
Bier, malt	<i>L. casei</i> : LACcas25 ₇ , LACcas29 ₇ , TMW 1.300 ₆
Coffee	<i>L. casei</i> : DSM20178 ₂ <i>L. rhamnosus</i> : DIAL401 ₅
Humans (saliva, dental caries, blood, urethra, faeces of infants and adults)	<i>L. casei</i> : LMG23516 ₁ <i>L. zeae</i> : N871 ₆ <i>L. paracasei</i> : DSM20020 ₂ , LMG9438 ₁ , LMG11459 ₁ , LMG23511 ₁ , LMG23518 ₁ , LMG23523 ₁ , LMG23538 ₁ , LMG23543 ₁ , LMG24098 ₁ , LMG24101 ₁ , LMG24132 ₁ , DBTA341 ₈ , DSM4905 ₂ <i>L. casei/paracasei</i> : N1611 ₆ , N421 ₆ , N441 ₆ , N761 ₆ , N17101 ₆ <i>L. rhamnosus</i> : DBTA861 ₈ , DBTC41 ₈ , N1711 ₆ , N1781 ₆ , N7151 ₆ , N941 ₆ , N951 ₆ , N831 ₆ , N2011 ₆ , N2091 ₆ , N20121 ₆ , N1321 ₆ , N221 ₆ , N261 ₆ , N8121 ₆ , N1731 ₆ , N11101 ₆ , N1311 ₆ , N211 ₆ , N1721 ₆ , N20101 ₆ , N20131 ₆ , N2021 ₆ , N251 ₆ , N1761 ₆ , N20111 ₆ , TMW 1.1538 ₆ , Mo21 ₆ , N8111 ₆ , N20141 ₆ , N1751 ₆
Unknown	<i>L. paracasei</i> : NRRL B-4561 ₉ , DSM5622 ₂ <i>L. rhamnosus</i> : NRRL B-1761 ₉ , NRRL B-4421 ₉ , DSM20021 ₂

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¹⁹ARS Culture (NRRL) Collection, United States Department of Agriculture, USA

710 **Table 2.**

Target microorganism	Primer name	Sequence (5'-3')	Amplicon size (bp)	Temperature of annealing (°C)	Reference
<i>L. casei</i>	casei Y2	TGCACTGAGATTTCGACTTAA CCCCTGCTGCCTCCCGTAGGAGT	290	53 °C	Ward and Timmins (1999)
<i>L. paracasei</i>	para Y2	CACCGAGATTCAACATGG CCCCTGCTGCCTCCCGTAGGAGT	290	53 °C	Ward and Timmins (1999)
<i>L. rhamnosus</i>	rham Y2	TGCATCTTGATTTAATTTTG ^{SEP} CCCCTGCTGCCTCCCGTAGGAGT	290	53 °C	Ward and Timmins (1999)
<i>L. rhamnosus</i>	dnaKRHf dnaKRHr	GAACAGCAGGGATCC GATCTTTCCGGTGTGA	235	58 °C	This study
<i>L. paracasei/casei</i>	dnaKCPf dnaKCPr	AAACTGTGCCCCGCGT GCGACGGGGTCTTTG	281	59 °C	This study
<i>L. casei</i>	dnaJPAf dnaJPAr	CGGCTGCGAACTGCATTA TTCCTGCTGGCACCCAAA	162	64 °C	This study

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713 **Table 3.** Comparison of the results obtained using the different techniques on 6 out of the 194
714 strains: inconsistent results.

	Strain	Original ID	ID Specie- Specific PCR (Ward and Timmins, 1999)	ID Multiplex (Ventura et al., 2003)	ID Specie-Specific PCR This work	ID HRM This work
Synonyms	LMG6904*	<i>L. casei</i>				
	DSM20011	<i>L. casei</i>				
	ATCC393	<i>L. casei</i>	<i>L. paracasei</i>	<i>L. casei</i>	<i>L. paracasei</i>	<i>L. paracasei</i>
	CCUG 21451	<i>L. zeae</i>				
Synonyms	DSM4905	<i>L. paracasei</i>				
	ATCC 1158	<i>L. casei</i>	<i>L. casei</i>	<i>L. paracasei</i>	<i>L. paracasei</i>	<i>L. casei</i>
	DBPZ0420	<i>L. rhamnosus</i>	<i>L. paracasei</i>	<i>L. rhamnosus</i>	<i>L. rhamnosus</i> <i>L. paracasei</i>	<i>L. paracasei</i>
	DBPZ0571	<i>L. paracasei</i>	<i>L. casei</i>	<i>L. paracasei</i>	<i>L. paracasei</i>	<i>L. casei</i>
	DBPZ0734	<i>L. rhamnosus</i>	<i>L. paracasei</i>	<i>L. rhamnosus</i>	<i>L. paracasei</i>	<i>L. paracasei</i>
	N2014	<i>L. rhamnosus</i>	<i>L. casei</i>	<i>L. rhamnosus</i>	<i>L. casei</i>	<i>L. casei</i>

*In bold it has been underlined the original name of the tested strain, as collected from the International collections
(see Table 1 and 4)

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Table 4. Final identification of the tested strains.

Origin	Identification
Raw and heat treated milk, yoghurt, milking machines	<i>L. casei</i> : <u>PIE5</u> ₃ <i>L. paracasei</i> : LMG9192 ₁ , DSM5622 ₂ , <u>PIE6</u> ₈ , <u>P2P3</u> ₃ , <u>DIALYac15</u> , <u>DIALDan15</u> <i>L. paracasei</i> subsp. <i>tolerans</i> : LMG9191 ₁ , DSM20258 ₂ <i>L. rhamnosus</i> : HA1114, PRA1525, <u>PIE4</u> ₃
Green, creamy and seasoned cheeses (Italian cheeses: Scamorza, Parmigiano Reggiano, Grana Padano, Spresa, Asiago, Montasio, Canestrato di Moliterno, Morlacco, Bellunese, Pecorino, Caciocavallo, Provolone, Emmenthal, Raclette de Savoie; Chinese and Tunisian cheeses)	<i>L. casei</i> : <u>CI4368</u> ₁₂ <i>L. paracasei</i> : LMG25880 ₁ , LMG25883 ₁ , LMG12164 ₁ , DBPZ0421 ₈ , DBPZ0422 ₈ , DBPZ0424 ₈ , DBPZ0434 ₈ , DBPZ0435 ₈ , DBPZ0450 ₈ , DBPZ0451 ₈ , DBPZ0472 ₈ , DBPZ0475 ₈ , DBPZ0476 ₈ , DBPZ0477 ₈ , DBPZ0478 ₈ , DBPZ0635 ₈ , DBPZ0733 ₈ , M266 ₈ , M268 ₈ , M299 ₈ , M308 ₈ , M348 ₈ , M354 ₈ , M359 ₈ , S1 ₈ , S3 ₈ , V3 ₈ , W11 ₈ , DSG03 ₈ , DSG05 ₈ , DSG07 ₈ , ESG10 ₈ , HSG09 ₈ , PSG06 ₈ , PSG09 ₈ , PSG10 ₈ , P71 ₉ , TH1229 ₉ , SP57 ₉ , L24 ₉ , TH406 ₉ , FSL43610, FSL45110, DBPZ0436 ₈ , <u>M290</u> ₈ , <u>M303</u> ₈ , <u>TMW1.1444</u> ₆ , <u>TMW1.1259</u> ₆ , <u>LACcas7</u> ₇ , <u>Cst7</u> ₁₁ , <u>3LC</u> ₁₁ , <u>DBPZ0718</u> ₈ , <u>CF143</u> ₁₂ , <u>R61</u> ₁₃ , <u>F17</u> ₁₃ , <u>N24</u> ₁₃ , H121 ₃ <i>L. rhamnosus</i> : M15 ₉ , O14 ₈ , PRA204 ₅ , PRA232 ₅ , PRA331 ₅ , DBPZ0420 ₈ , <u>DBPZ0428</u> ₈ , DBPZ0430 ₈ , DBPZ0445 ₈ , DBPZ0446 ₈ , DBPZ0448 ₈ , DBPZ0449 ₈ , FSG01 ₈ , CI2301 ₂ , CI43621 ₂ , CF13501 ₂ , CF3771 ₂ , D441 ₃ , H251 ₃ , 5A9T ₉ , 5D9T ₉ , L9 ₉ , L47 ₉ , <u>LACcas13</u> ₇ , <u>M335</u> ₈ , <u>M307</u> ₈
Fermented sausages	<i>L. paracasei</i> : <u>CTC1675</u> ₁₄ <i>L. rhamnosus</i> : CTC16761 ₄ , 22201 ₅
Sourdoughs	<i>L. paracasei</i> : DBPZ0561 ₈ , DBPZ0572 ₈ , Q2 ₈ , Q4 ₈ , I1 ₄ , I21 ₆ , <u>DBPZ0563</u> ₈ , <u>DBPZ0564</u> ₈ , <u>DBPZ0579</u> ₈ , <u>I3</u> ₁₆
Wine, must and cellar equipment's	<i>L. casei</i> : <u>B166</u> ₁₇ <i>L. paracasei</i> : LMG119611, LMG119631, LMG137171, LMG137311, B0611 ₇ , <u>B082</u> ₁₇ , <u>B083</u> ₁₇ , B0851 ₇ , <u>B087</u> ₁₇ , B1611 ₇ , B1691 ₇ , B1711 ₇ , B1741 ₇ , B1951 ₇ , B1961 ₇ , B3501 ₇ <i>L. rhamnosus</i> : <u>B084</u> ₁₇ , <u>B086</u> ₁₇ , <u>B163</u> ₁₇ , <u>B164</u> ₁₇ , <u>B167</u> ₁₇ , <u>B168</u> ₁₇ , <u>B170</u> ₁₇ , <u>B172</u> ₁₇ , <u>B173</u> ₁₇ , <u>B175</u> ₁₇ , <u>B179</u> ₁₇
Bier, malt	<i>L. paracasei</i> : <u>LACcas25</u> ₇ , <u>LACcas29</u> ₇ , <u>TMW 1.300</u> ₆
Coffee	<i>L. casei</i> : DSM20178 ₂ <i>L. rhamnosus</i> : DIAL401 ₅
Humans (saliva, dental caries, blood, urethra, faeces of infants and adults)	<i>L. casei</i> : LMG235161, <u>N87</u> ₁₆ , <u>N811</u> ₁₆ <i>L. paracasei</i> : DSM20020 ₂ , LMG94381, LMG114591, LMG235111, LMG235181, LMG235231, LMG235381, LMG235431, LMG240981, LMG241011, LMG241321, DBTA341 ₈ , <u>N161</u> ₁₆ , <u>N42</u> ₁₆ , <u>N44</u> ₁₆ , <u>N76</u> ₁₆ <i>L. rhamnosus</i> : DBTA861 ₈ , DBTC41 ₈ , N1711 ₆ , N1781 ₆ , N7151 ₆ , N941 ₆ , N951 ₆ , N831 ₆ , N2011 ₆ , N2091 ₆ , N20121 ₆ , N1321 ₆ , N221 ₆ , N261 ₆ , N8121 ₆ , N1731 ₆ , N11101 ₆ , N1311 ₆ , N211 ₆ , N1721 ₆ , N20101 ₆ , N20131 ₆ , N2021 ₆ , N251 ₆ , N1761 ₆ , N20111 ₆ , TMW 1.1538 ₆ , Mo21 ₆ , <u>N1710</u> ₁₆ , N1751 ₆
Unknown	<i>L. paracasei</i> : NRRL B-4561 ₉ , DSMZ 5622 ₂ <i>L. rhamnosus</i> : NRRL B-1761 ₉ , NRRL B-4421 ₉ , DSMZ20021 ₂

*Strains with uncertain identification: LMG 6904₁, DSM4905₂, DBPZ0420₈, DBPZ0571₈, DBPZ0734₈, N20141₆

The strains underlined in red didn't result to belong to the *L. casei* group; The identification of the strains underlined in black was in disagreement with the original identification, the new identification has been reported.

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